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Analysis of gene regulation in growing pollen tubes of angiosperm and gymnosperm species using microprojectile bombardment

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A microprojectile based transient expression assay was used to investigate the functional conservation of gene regulatory mechanisms in the male gametophytes of an angiosperm (*Nicotiana tabacum*) and two gymnospermous (*Picea abies* and *Pinus pinaster*) species. The activities of two angiosperm gene promoters, which have previously shown to be either preferentially expressed in the male gametophyte (*lat*52) or highly expressed in both the sporophyte and male gametophyte (*Act*1), were analysed. The results showed that in *P. abies* and *P. pinaster*, activity of the *Act*1 promoter was significantly higher than the activity of the *lat*52 promoter, while the converse was observed in *N. tabacum*. Detailed analysis of *lat*52 5' promoter deletions demonstrated that although the minimal –67 bp *lat*52 core promoter was active at low levels in all three species, upstream regulatory elements conserved among several pollen-expressed genes, including the PBI element, were not functional in *P. abies* and *P. pinaster*. These results suggest that both taxa-specific and conserved regulatory mechanisms operate to control gene expression during pollen germination and tube growth.

Key words – Actin, maritime pine, microprojectile bombardment, *Nicotiana tabacum*, Norway spruce, *Picea abies*, *Pinus pinaster*, pollen, promoter deletions, tobacco, transient expression.

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Introduction

The relatively simple developmental pathway, accessibility for isolation and in vitro manipulation highlights the male gametophyte (or pollen grain) as an experimental system for studies on the molecular mechanisms of cellular differentiation. An important technology that has greatly facilitated the direct analysis of gene regulatory mechanisms in pollen is particle bombardment (Klein et al. 1987), which provides a facile and efficient method for delivery of DNA into pollen (Twell et al. 1989, 1991b). All currently published studies using this technology have been carried out with pollen of angiosperms, including both monocotyledonous and dicotyledonous species (Twell et al. 1989, 1991a, b, Hamilton et al. 1992, van der Leede-Plegt et al. 1992, Nishihara et al. 1993), which has led to the identification of conserved regulatory elements among several genes which are preferentially expressed in maturing pollen (Twell et al. 1991b). Thus, established technologies have not been applied to investigate gene regulation in species of gymnosperms, such that the extent to which pollen-expressed genes and their regulatory mechanisms are conserved between angiospermous and gymnospermous species is unknown.

In the majority of angiospermous species, including *Nicotiana tabacum*, the haploid microspores undergo a single asymmetric mitotic division prior to dehiscence, thus forming a pollen grain with a large vegetative cell and a small generative cell (Brewbaker 1967). In contrast, in *Picea* spp. (Owens et al. 1979, Dawkins and Owens

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1993) and *Pinus* spp. (Stanley et al. 1974), the microspore nucleus undergoes several mitotic divisions before pollen shed, producing a mature pollen grain containing two non-functional prothallial cells, a large vegetative cell, and a smaller generative cell. In *Picea* spp., the generative cell divides once more to produce a body cell and a stalk cell (Dawkins and Owens 1993). In both angiospermous and gymnospermous species the pollen tube growth appears to be largely under the control of a functionally homologous, vegetative cell. However, pollen of angiosperms and gymnosperms have differing requirements to achieve fertilization. Such characteristics are reflected in the in vitro germination behaviour of both pollen types.

In tobacco, using optimised germination and in vitro growth conditions, the pollen tube tip emerges within 30–60 min of hydration and tube growth continues for approximately 12 h, reaching a maximum length of about 500 μm (Hoffman et al. 1988). In contrast, pollen of spruce and pine germinates slowly (only after 15 h hydration in vitro), but pollen tube elongation continues for up to 72 h, reaching mean pollen tube length of 610 ± 45 μm (Martinussen et al. 1994). Thus, it may be expected that certain gene regulatory activities of the vegetative cell of angiospermous and gymnospermous pollen during germination and tube growth would be conserved, although these activities would necessarily be maintained over a longer time period in pollen of gymnosperms.

The aim of this study was to investigate the conservation of gene regulatory mechanisms in tobacco, spruce and pine pollen using microprojectile bombardment from a functional standpoint. This study included comparative experiments with the well characterized male gametophyte-specific *lat*52 promoter from tomato (Twell et al. 1990) and the rice actin promoter *Act*1, which is highly active in both sporophytic and gametophytic cells (McElroy et al. 1990). Our results demonstrate that the previously described (Twell et al. 1991b) *cis*-regulatory elements PBII and PBI function in tobacco pollen, but are not recognised in pollen of spruce and pine. Furthermore, based upon the similar activity of the *Act*1 promoter in pollen of all three species regulatory features of the *Act*1 promoter appear to be more highly conserved.

Materials and methods

Preparation and germination of pollen

Norway spruce [*Picea abies* (L.) Karst.] pollen was collected at Biri forest nursery, Norway, in spring 1992, dried and stored in plastic bottles at −70°C. Tobacco (*Nicotiana tabacum* cv. Samsun) pollen was harvested from greenhouse grown plants and stored in 1.5-ml microfuge tubes at −70°C. Pollen of maritime pine (*Pinus pinaster*) was harvested from Leicester Botanical Gardens in spring 1992, dried and stored in 10-ml polypropylene tubes at −70°C. The same batches of pollen were used for all experiments. Spruce and pine pollen were germinated in Muren's mineral medium (Muren et al. 1979) while pollen of tobacco was germinated in Tupy's medium (Tupy et al. 1991). Approximately 25 mg dry pollen was suspended in liquid medium and adjusted to a density of 1×10^6 pollen grains ml^{-1}. The pollen suspension (400 μl) was pipetted onto a nylon membrane (Hybond N), overlaying a sterile Whatman No. 1 filter paper on the surface of solidified germination media (0.8% w/v agar) in a 9-cm petri dish.

Plasmids

For the analysis of gene expression in pollen, microprojectile bombardment was utilized to introduce two different promoters: *lat*52 (Twell et al. 1990) and *Act*1 (McElroy et al. 1990, 1991, Zhang et al. 1991). These were linked to the *E. coli* β-glucuronidase (GUS) gene (*gus*A) in plasmids pLAT52-7 and pAct1-D, respectively. The *lat*52 promoter region −492 to +110 bp was fused to the firefly luciferase (LUC) gene (*luc*) in plasmid pNBL52-5. 5' Deletion derivatives corresponding to *lat*52 promoter positions −225 bp, −145 bp, −100 bp and −17 bp were subcloned using the restriction enzyme sites *SalI*/*Nco*I from a plasmid series in which these were linked to the *gus*A coding DNA described in Twell et al. (1991b). The *lat*52 deletion derivative at −67 bp was created using the 5' specific oligonucleotide primer 5' GCACGTCGACTAATAGCTCCACCATAT 3' containing a 5' *Sal*I restriction site and a 3' primer present in the *gus*A coding DNA, 5' CGATCCAGACTGAATGCC 3'. The template used for PCR was pLAT52-7 (Twell et al. 1990) which resulted in the synthesis of the −67 to +110 bp *lat*52 fragment. This fragment was cloned into pNBL52-5 using the restriction enzymes sites *Sal*I and *Nco*I. The −67 deletion derivative was completely sequenced and contained the same sequence as the template *lat*52 promoter present in pLAT52-7. Large scale plasmid preparations were carried out according to Frei et al. (1985). The concentrations of the plasmid stock solutions were equalized to each other by comparing the relative intensity of supercoiled DNA on an ethidium bromide stained 0.8% agarose gel. After equalization, the same plasmid stock solutions were used for all the experiments.

Preparation of microprojectiles and bombardment

Plasmid DNA was precipitated onto M-10 (average diameter 0.9 μm) tungsten microprojectiles and bombardments were carried out according to Twell et al. (1989, 1991a) using a gunpowder charge-driven particle gun described by Klein et al. (1988). Bombardment was performed within 30 min of suspending the dry pollen in germination medium. Seven μg test plasmid, and in the deletions experiment 2 μg reference plasmid, were precipitated onto ca 1 250 μg tungsten particles in a solution containing $CaCl_2$ (ca 360 m*M*) and spermidine (ca 14.3 m*M*). Unbombarded pollen and pollen bombarded with

uncoated particles were used as negative controls in all experiments.

Assay of GUS and LUC activities

Petri dishes with bombarded pollen were sealed with Parafilm (American National Can_{TM}) and incubated at 25°C under coolwhite fluorescent light (Thorn 58 W tubes, photon flux density 100 μmol m^{-2} s^{-1}). Spruce and pine pollen were harvested for transient expression after 24 h, whilst tobacco pollen was harvested 16 h after bombardment. Pollen was scraped from the nylon filter with a microscope slide and ground in 500 μl extraction buffer consisting of 0.1 *M* potassium phosphate buffer, pH 7.5, 1 m*M* dithiothreitol. Pollen extracts were centrifuged at 16 400 *g* (14 000 rpm) for 5 min in a microfuge at room temperature and supernatants assayed for GUS and LUC activity as previously described by Jefferson et al. (1987) and Ow et al. (1986), respectively, with modifications by Twell et al. (1991a). To 400 μl 1 m*M* MUG (4-methylumbelliferyl-β-D-glucuronide, in 50 m*M* $NaH_2PO_4 \cdot 2\ H_2O$, 1 m*M* EDTA, 10 m*M* β-mercaptoethanol, 0.1% v/v Triton X-100) incubated at 37°C, 100 μl pollen extract were added. The reaction was stopped by adding 100 μl reaction mixture to 100 μl GUS stop buffer (0.2 *M* Na_2CO_3) and GUS activity was assayed fluorimetrically in a Perkin Elmer luminescence spectrometer LF50 equipped with a microplate reader. In a standard microtitre well 1 pmol 4-MU is equivalent to 0.657 fluorimetric units (FU). LUC activities in 20 μl pollen extracts were measured in a luminometer (Berthold model CliniLumat), and background activity (counts s^{-1}) from the substrate was subtracted from LU values. GUS and LUC activities are expressed as fluorimetric units and light units per bombardment, respectively, with a standard number of 1×10^6 pollen grains used per bombardment.

Results

In order to compare transient gene expression in the different samples, the same batches of pollen, one from each species, were used for all the experiments. Pollen viability was measured by in vitro germination on optimal medium for the three species. The portion of viable pollen in the different samples was approximately the same, with 85% of pine, 92% spruce and 95% of tobacco pollen germinating after 40, 24 and 16 h, respectively.

Timecourse of *lat*52- and *Act*1-directed gene expression

To establish optimal conditions required to support transient gene expression in tobacco, spruce and pine pollen, *lat*52 promoter activity was assayed at various time points following bombardment with plasmid pNBL52-5. This plasmid contained the *lat*52 promoter region −492 to +110 bp linked directly to the ATG initiator codon of the luciferase (*luc*; Ow et al. 1986) reporter gene. In tobacco pollen, maximum levels of luciferase (LUC) activity (~3.6×10^8 light units per bombardment) were observed as a rather sharp peak 12 h after bombardment (Fig. 1a). In contrast, spruce pollen expressed maximal levels of LUC activity which (~1.7×10^6 light units per bombardment) were approximately 208-fold lower than in tobacco, but were maintained at similar levels from 12 to 48 h after bombardment (Fig. 1b). The time course of *lat*52 directed LUC activity in pine pollen observed after bombardment with pNBL52-5 showed a very similar profile to that of spruce (data not shown). There was no detectable background LUC activity in pollen, either unbombarded or bombarded with uncoated tungsten microprojectiles, of tobacco, spruce (Fig. 1b) and pine. Light units per bombardment measured in these negative controls was less than zero after subtracting background light units derived from the sample counted in the absence of the substrates Mg^{2+}, ATP and luciferin. These data indicate that the *lat*52 promoter is activated in pollen of both tobacco and gymnospermous pollen, but in gymnospermous pollen the *lat*52 promoter is active only at a low level. Furthermore, the processes which mediate de

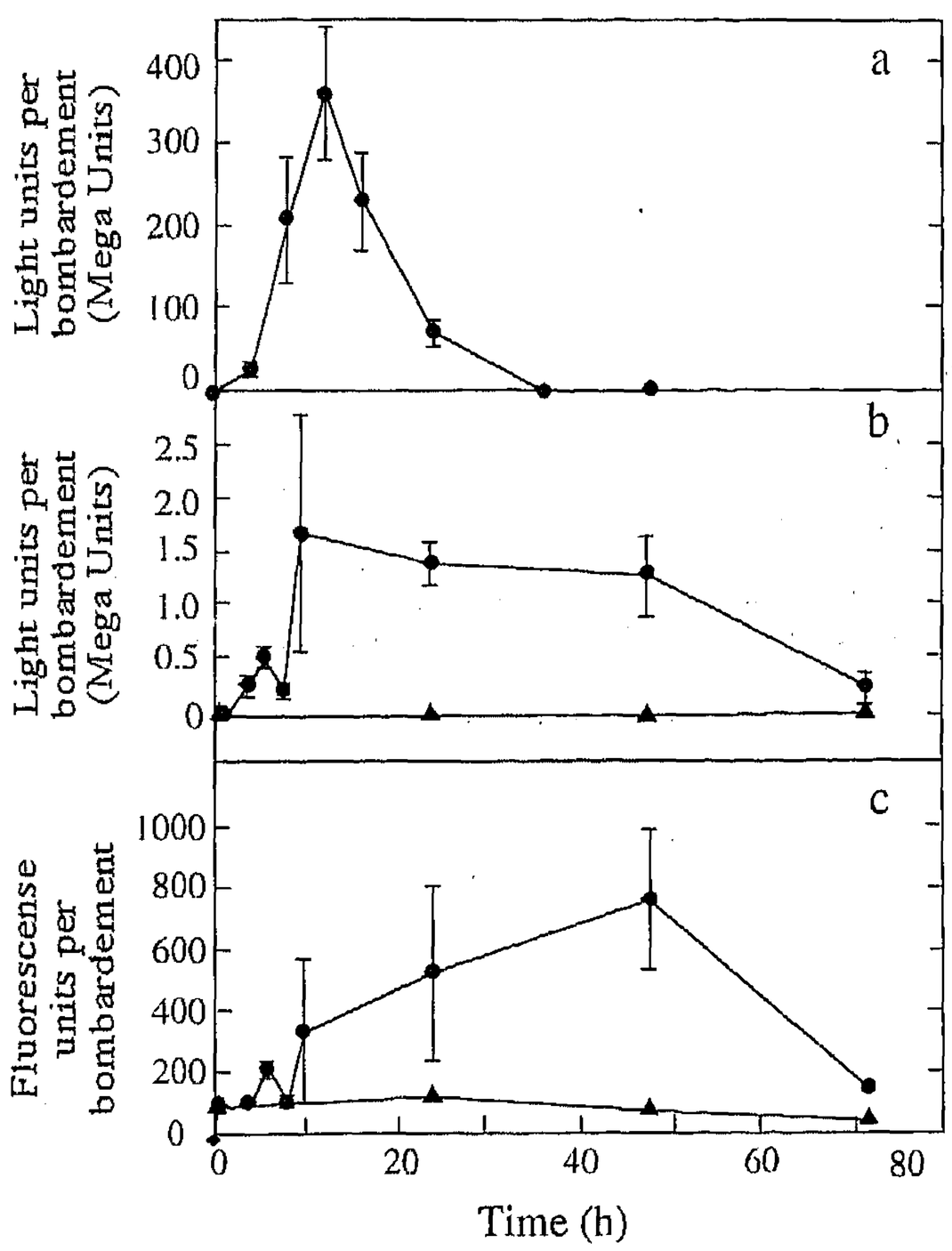

Fig. 1. Transient gene expression of the construct pNBL52-5 (*lat*52-*luc*) bombarded into (a) *N. tabacum* and (b) *P. abies* pollen, assayed at different times after bombardment. (c) Transient gene expression of the construct pAct1-D (*Act*1-*gus*A) in *P. abies* pollen during germination from 0 to 72 h after bombardment. LUC and GUS activities are expressed as light units or fluorescence units, respectively, per bombardment of 1×10^6 pollen grains. The means and SE values (bars) of at least 3 individual experiments, with 3 replicates per treatment, are shown (●). The baselines show GUS or LUC activity in pollen bombarded with tungsten particles without DNA (▲).

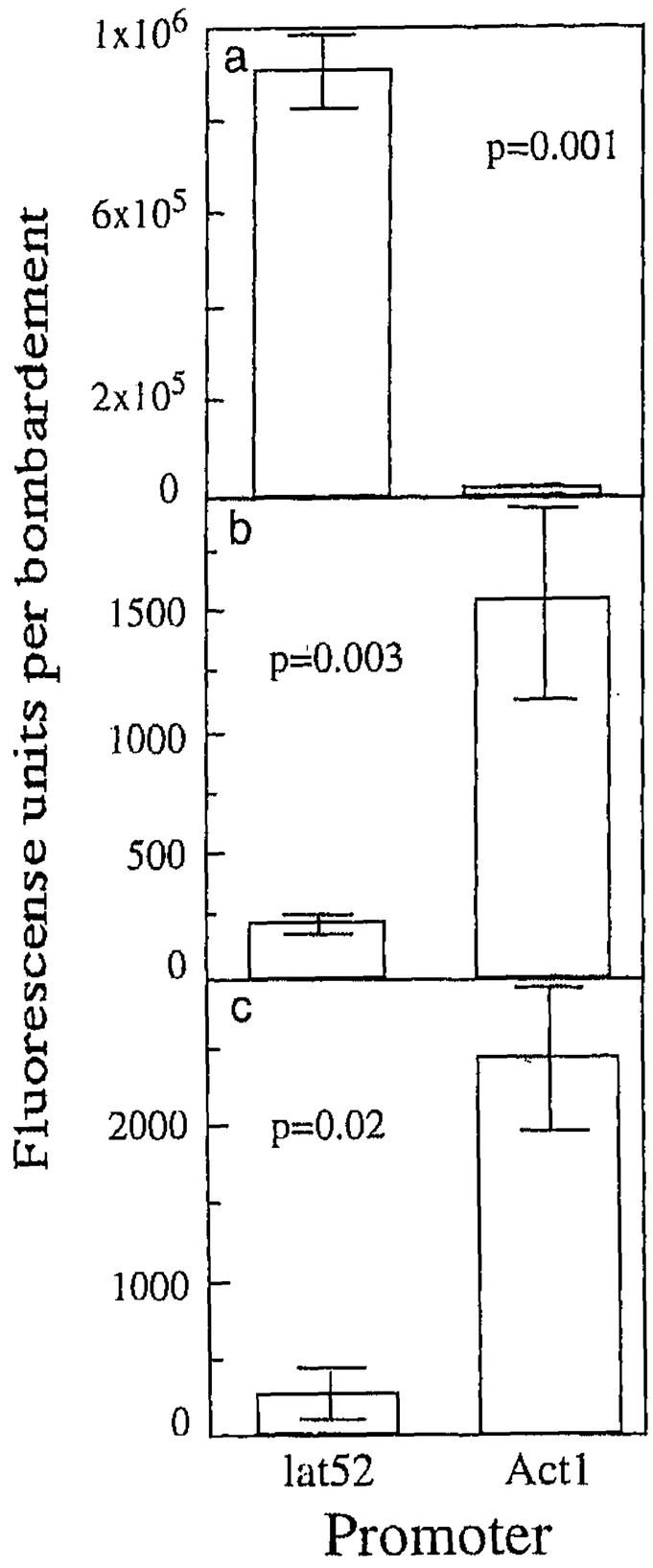

Fig. 2. Relative activities of the *lat*52 and *Act*1 promoters in pollen of *N. tabacum*, *P. abies* and *P. pinaster*. The histograms show transient gene expression from the plasmids pLAT52-7 (*lat*52-*gus*A) and pAct1-D (*Act*1-*gus*A) after bombardment into pollen of (a) *N. tabacum*, (b) *P. abies* and (c) *P. pinaster*. GUS activity was measured after 16 h for *N. tabacum* and 24 h for *P. abies* and *P. pinaster* and represents the fluorescence units per bombardment of 1×10^6 pollen grains. The means and SE values of 3 experiments, with 6 replicates per treatment, are shown. Statistical *t*-test shows significant differences.

novo *lat*52 promoter activity appear to be rapidly activated in all three species following hydration, but are maintained over a longer time period in spruce and pine than in tobacco. This may result from the more rapid cessation of tube growth in tobacco pollen, which is observed under in vitro growth conditions (Tupy et al. 1991).

The activity of the *Act*1 promoter in germinating pollen was investigated using β-glucuronidase (GUS; Jefferson et al. 1987) as a reporter following bombardment of pollen with the plasmid pAct1-D (McElroy et al. 1991). These experiments showed that the pattern of accumulation of *Act*1 directed GUS activity in pollen tubes of tobacco was very similar to that of the *lat*52 promoter using either GUS or LUC as a reporter (data not shown). In contrast, in spruce pollen, *Act*1-directed GUS activity increased steadily from 12 to 48 h, and then declined to a low level 72 h after bombardment (Fig. 1c). Comparison of the time courses indicate that *lat*52-directed expression was at considerably higher levels in tobacco than in spruce and pine pollen, and that *Act*1-directed expression was low but at similar levels in all three species. Levels of GUS activity directed by the *Act*1 promoter in spruce were at least 20-fold above background levels observed in pollen bombarded with uncoated microprojectiles. *lat*52-Directed GUS activity levels in spruce were at least 3-fold above the negative controls after 24 h of incubation. In pine, GUS activity levels at 24 h were 25- and 240-fold above background directed by the *lat*52 and the *Act*1 promoters, respectively.

Relative activities of *lat*52 and *Act*1 promoters

The relative activities of the *lat*52 and *Act*1 promoters were compared directly in pollen of all three species after bombardment with the plasmids pLAT52-7 (Twell et al. 1989) and pAct1-D (McElroy et al. 1991), which contain the respective promoter regions fused to the *gus*A gene. Maximum levels of transient expression were observed 16 h after bombardment in tobacco and 24 h after bombardment in spruce and pine pollen as determined in the time course experiments described above. Levels of *lat*52 directed GUS activity in tobacco pollen were approximately 50-fold higher than those directed by the *Act*1 promoter (Fig. 2a). In contrast, *Act*1 directed GUS activities in spruce and pine were approximately 6- to 10-fold higher than that directed by the *lat*52 promoter, respectively (Fig. 2b,c). Based upon the absolute levels of GUS activity shown in Fig. 2, the *Act*1 promoter appears to be 12- and 8-fold more active in tobacco (ca 19 000 fluorimetric units per bombardment) than in spruce (ca 1 500) and pine (ca 2 400) pollen, respectively. Since it was not possible to control internally for the efficiency of DNA delivery between tobacco and gymnospermous pollen, the percentage of blue staining (GUS positive) grains were counted in three independent bombardment experiments with tobacco and spruce. No differences were noted in the percentage of GUS positive pollen in these experiments which varied between 0.2 and 1% in both species. These data suggest that there was a similar efficiency of DNA delivery in pollen of both species, although the efficiency of DNA delivery clearly varied between independent bombardments. Taken together with the absolute levels of GUS activity directed by the *lat*52 promoter, these data suggest that the *lat*52 promoter is at least 3 700-fold more active in tobacco (ca 10^6 fluorimetric units per bombardment) than in spruce (240) pollen. The *lat*52 promoter appears to be expressed at similar levels in spruce (240) and in pine (256); also the *Act*1 promoter showed similar activities in both gymnospermous species, spruce (ca 1 500 fluorimetric units per bombardment) and pine (ca 2 400).

5' Deletion analysis of *lat*52 promoter

To investigate further the basis of the differential activity

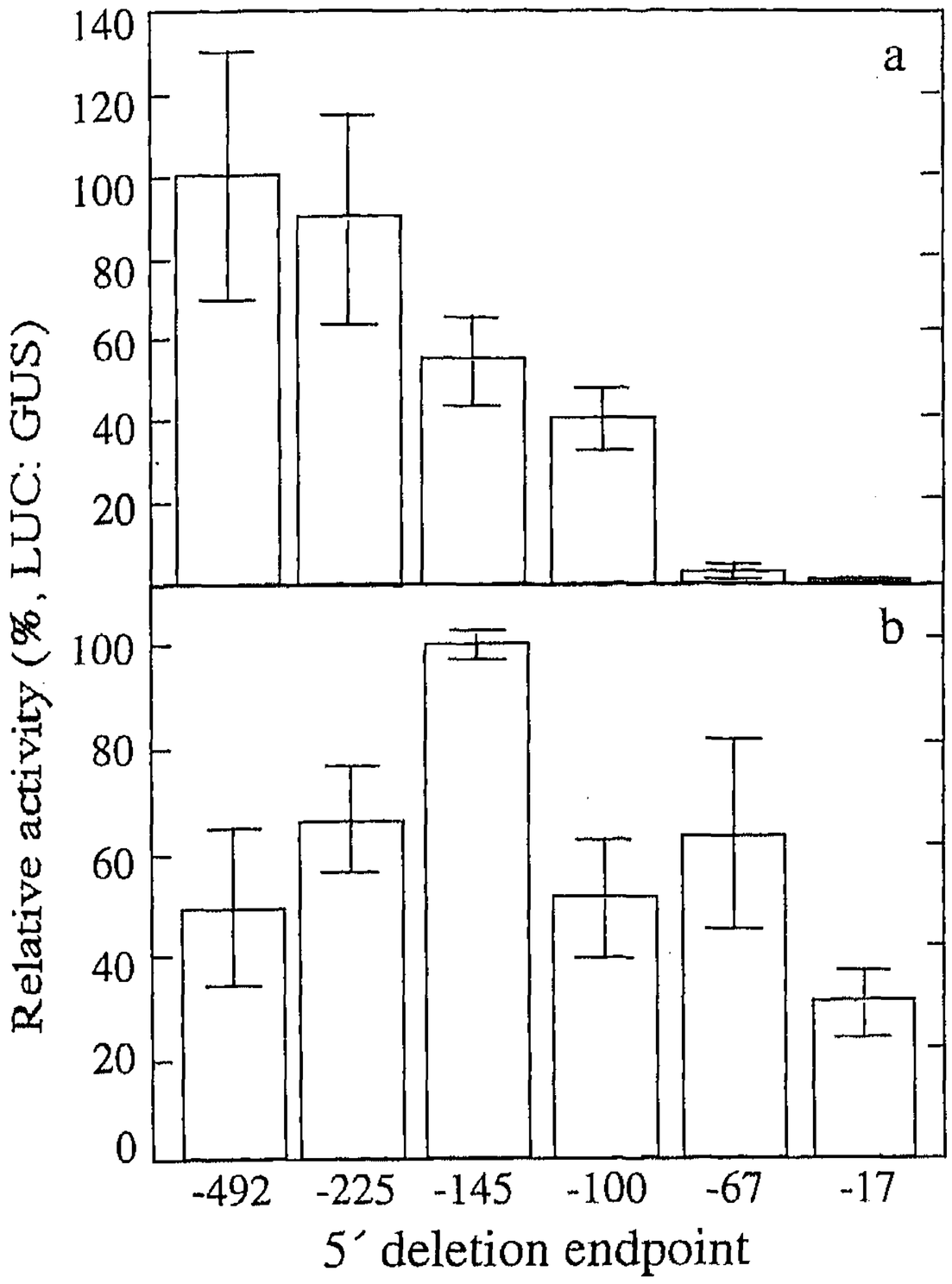

Fig. 3. Relative activities of 5' deletion-derivatives of the *lat*52 promoter in pollen of *N. tabacum* and *P. abies*. Seven µg of the test *lat*52-*luc* plasmid containing a 5' deletion of the *lat*52 promoter were co-bombarded with 2 µg of the reference plasmid pLAT52-7 (*lat*52-*gus*A) into pollen of (a) *N. tabacum* and (b) *P. abies*. Each test plastmid was bombarded separately. Relative activities of the test plasmids were calculated from the ratio LUC:GUS activity. The means and SE values of 3 individual experiments, with 3 replicates per treatment, are shown.

of the *lat*52 promoter in pollen of the three species, the activities of a series of 5' deletions of the *lat*52 promoter linked to the *luc* gene were investigated following bombardment of pollen of all three species. Co-bombardment of a reference plasmid containing the *lat*52 promoter linked to the *gus*A gene (pLAT52-7) provided an internal control for variability in the efficiency of DNA delivery between bombardments (Twell et al. 1991b). Thus, the relative promoter activity of the deletion constructs was calculated as the relative LUC:GUS ratio, and presented as relative activity (percentage of maximum value, Fig. 3). In tobacco pollen *lat*52 promoter activity decreased 2-fold when deleted from −492 to −145 bp and decreased a further 10-fold when deleted from −100 to −67 bp (Fig. 3a). These promoter regions correspond to regulatory regions containing the *cis*-regulatory elements PBII and PBI, respectively, which have been previously described (Twell et al. 1991b). Further deletion of the *lat*52 promoter from −67 to −17 bp reduced promoter activity a further 4-fold, such that the activity of the −17 deletion was not above that of a promoterless control plasmid containing the *luc* gene (Fig. 3a). Similar analysis in spruce (Fig. 3b) and pine (data not shown) pollen showed a different pattern. In both spruce and pine pollen there was a decrease in *lat*52 promoter activity upon deletion from −67 to −17 bp. However, activity of the −492 bp construct was not increased significantly above that of the −67 bp deletion ($P > 0.05$). Furthermore, deletion of the regulatory elements including the PBI and PBII elements did not lead to a reduction of the *lat*52 promoter activity. This suggests that although the core *lat*52 promoter region −67 to +110 bp functions in pollen of all three species, upstream regulatory elements of the *lat*52 promoter are not recognised in spruce and pine pollen.

Discussion

The activity of the pollen-specific *lat*52 promoter and the gametophytic-sporophytic promoter *Act*1 were investigated in pollen of tobacco, and in two gymnospermous species, spruce and pine, using a transient expression assay involving microprojectile bombardment. The results showed that in spruce and pine the relative activity of the *Act*1 promoter was significantly higher than that of the *lat*52 promoter ($P < 0.05$), while the converse was observed in tobacco ($P < 0.05$). The *lat*52 promoter was 190- to 750-fold more active in tobacco than in the gymnosperms taking into consideration assays performed using both *gus*A and *luc* reporter genes (Figs 1 and 2). The results indicate that the *lat*52 promoter functions differently in germinating pollen of these gymnosperms when compared with tobacco. Thus, the regulation of the *lat*52 promoter does not appear to be conserved between tobacco and the gymnosperms spruce and pine. Recent results in which the *lat*52 promoter was reported to be inactive in pollen of monocotyledons such as maize and calla lily (Twell et al. 1991a), and in that of *Lilium longiflorum* (van der Leede-Plegt et al. 1992) using the bombardment technique, extend this finding and further suggest that transcription factors that mediate *lat*52 activity have not been conserved even among angiosperm species.

Previous analyses have demonstrated that the *lat*52 promoter functions at high levels in pollen of tomato, tobacco and *Arabidopsis* in both stably transformed plants and in transient bombardment experiments (Twell et al. 1990, 1991b), which identifies a subgroup of dicotyledonous species with bicellular and tricellular pollen that have conserved mechanisms regulating late pollen gene expression. Based upon the low level of activity of the *lat*52 promoter in spruce and pine pollen we postulate that mechanisms regulating pollen gene expression in angiospermous and gymnospermous pollen have evolved along diverse pathways. Our investigations of the activity of a series of *lat*52 promoter deletion derivatives support this hypothesis and precisely identify regulatory components, such as the PBI element within the region −100 to −67 bp, are not recognised in pollen of spruce and pine. In contrast, the activity of the *Act*1 promoter was similar in all three species, which suggests that mechanisms

regulating actin gene expression in the gametophyte may be conserved. These results further support the contention that the regulation of genes, such as actin, whose functions are necessarily conserved are more likely to be conserved, while the regulation of genes that are species or taxa-specific, such as *lat*52, are likely to have developed novel and/or divergent mechanisms. However, at this point we cannot rule out the possibility that promoters such as that of *Act*1 derived from monocotyledonous species are particularly active in pollen of gymnosperms compared with those derived from dicotyledonous species, such as *lat*52. From an evolutionary standpoint this is unlikely, but this could be tested directly given the availability of the *lat*52 homologous genes from maize (Hamilton et al. 1989) and rice (Zou et al. 1994).

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